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Studies of the biological function and structure of casein micelles, and future implications

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Abstract: At the heart of the milk system are the colloidal casein–calcium–transport complexes termed the casein micelles. Despite extensive research in more than half a century, accomplished by the application of a wide range of physical techniques such as light, neutron, and X-ray scattering, and Electron Microscopy (EM), the molecular details of casein micelles and the main contributing forces that stabilize them in milk remain controversial and still sustain interest and effort in the dairy science research community. Among various proposed molecular models, two main conflicting theories about the internal structure of casein micelles have emerged. One places the emphasis on the four main protein constituents, α_{s1}-, α_{s2}-, β- and κ-casein in the micelles, while the other proposes that inorganic calcium phosphate nano-clusters are the dominant players in holding the micelles together. In this chapter, casein micelles are examined in the light of recent advances in understanding protein–protein interactions (associations) and protein structure–function relationships. The biological significance of casein micelles, in relation to their unique construct, allows for an efficient transit through the mammary secretory apparatus and this is critically assessed, in addition to the existing overwhelming amount of evidence supporting the argument that proteinaceous complexes act as the formative agents in the synthesis of casein micelles in mammary tissue; in other words, that protein–protein interactions are paramount in the formation and stabilization of casein micelles.

Key words: casein micelles, structure, protein–protein interaction, biological function, TEM.

6.1 Introduction

The appearance of milk is that of a creamy white fluid. The lubricity and taste of milk are based upon three unique biological structures: the colloidal calcium–protein complexes, often termed as casein micelles, the milk fat globules with their limiting membrane, and the milk sugar, lactose. The complexity of these structures is necessitated by the fact that milk, in most species, is in essence predominantly water. It is the accommodation of these

ingredients to an aqueous environment that forms the basis for the structure of milk at the molecular level and calls for the unique secretory process: milk synthesis (Patton, 2004).

It is generally believed (Farrell, 1999; Farrell *et al.*, 2003a) that the casein micelles are responsible for transporting and delivering the otherwise insoluble inorganic calcium and phosphate, indispensable nutrients for the neonates. Although these colloids have been the subject of extensive research for more than half a century (Noble and Waugh, 1965; Waugh *et al.*, 1970; Payens, 1979; Schmidt, 1982; Griffin and Roberts, 1985; Farrell and Thompson, 1988; Holt, 1992; Hansen *et al.*, 1996; Walstra, 1999; Horne, 2002; Tuinier and de Kruif, 2002; Qi, 2007; McMahon and Oommen, 2008; Fox and Brodkorb, 2008), the structural details of the casein micelles on the molecular level remain elusive and still sustain interest and effort (Farrell *et al.*, 2006a; Qi, 2007; Fox and Brodkorb, 2008; McMahon and Oommen, 2008). Past biochemical and physical studies of these colloids have focused on the size and properties of the colloids, their protein and mineral composition, and the central building block of the micelles. Conflicting views on the structure of the casein micelles have arisen from various interpretations of the core data published so far. In the light of recent advancements in the field of structural biology and systems biology, this manuscript will focus on the implications of protein–protein interactions in an attempt to discern the biologically competent route for the formation, and possibly stabilization, of the casein micelles.

The central theme of protein primary sequence to structure to function that dominated the field of molecular biology and structural biology for the past few decades is currently being challenged and debated by many (Meier *et al.*, 2008; Szilagyi *et al.*, 2008; Wright and Dyson, 1999). It has recently been recognized that the family of natively unfolded (Weinreb *et al.*, 1996) or intrinsically unstructured (Wright and Dyson, 1999; Dyson and Wright, 2002, 2005; Tompa, 2002) or intrinsically disordered proteins (Dunker *et al.*, 2001; Uversky, 2002; Bracken *et al.*, 2004; Radivojac *et al.*, 2007) is rapidly expanding. They are often found in eukaryotes (Dunker *et al.*, 2008) and are believed to be involved in multiple-partner binding sites, post-translational modifications, and alternative splicing that may lead to protein aggregation and amyloidogenesis (Uversky, 2008). These proteins, or sometimes long stretches of sequence in one protein, have been categorized as 'non-folding' and contain different amino acid sequences from those that fold into globular 3D structures. Caseins have been implicated as a prime example of this class of proteins (Farrell *et al.*, 2006b,c).

6.2 Brief review of proposed models for casein micelles

6.2.1 Protein composition of skim milk

The dominant protein constituents in skim milk are present in an aggregate form: the casein micelle (Fig. 6.1). This unique supramolecular aggregate

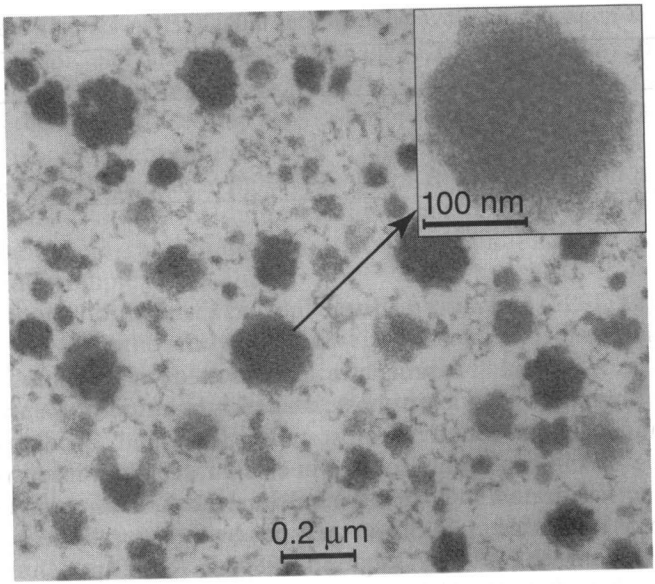

Fig. 6.1 Transmission Electron Micrograph of bovine milk casein micelles fixed with glutaraldehye and stained with uranyl acetate and lead citrate (pH 7.0). Inset at the upper right shows an enlarged single micelle.

imparts the opalescence characteristic of skim milk. The chief function of the micelle is to fluidize the casein molecules and solubilize the calcium and phosphate (Farrell and Thompson, 1988). In general, when milks that contain >2% protein are analyzed, the accompanying inorganic phosphate and calcium levels found would, by themselves, yield insoluble precipitates (apatite or brushite, depending upon the pH). Conversely, in the absence of these salts, the casein components, as a result of their open structures, would have a high viscosity. The formation of the casein colloidal complexes, micelles, averts these two problems.

Four major casein components in cows' milk, α_{s1}-, α_{s2}-, β- and κ-casein, have been well characterized in the past (Swaisgood, 2003). Caseins have been found to be homologous to these proteins in their gene and primary protein sequences in all species examined to date (Farrell *et al.*, 2004). However, the proportions of the various caseins vary widely. For example, β-casein is the primary casein in human milk, and in goats' milk it comprises 40% to 50% of the casein. In goats' milk there is also a high degree of variance in casein proportions in different animals, which appears to be genetically controlled (Table 6.1). Despite the variations in casein components, all species competently form colloidal casein micelles for the transport of calcium and phosphate. At the ultra-structural level, particularly in terms of the average size, the casein micelles of most species such as goat

Table 6.1 Casein distribution (%) in various milks

Milk	α_{s1}-	α_{s2}-	β-	κ-
Goat	5 to 17	6 to 20	50	15
Cow	38	10	40	12
Human	Trace	Trace	70	27

(Pierre *et al.*, 1998) and mouse (Burgoyne and Duncan, 1998) appear to be quite similar.

The α_{s1}-, α_{s2}-, and β-caseins are precipitated by calcium (calcium-sensitive) binding to their phosphoserine residues at the concentrations of protein and calcium found in most milks (Fox and Brodkorb, 2008). However, κ-casein is not only soluble in calcium (calcium-insensitive), but also interacts with and stabilizes the calcium insoluble caseins to initiate formation of the stable colloidal state. It has been generally recognized by studies (Hill and Wake, 1969; Dalgleish, 1980; de Kruif and Zhulina, 1996; Carroll and Farrell, 1983). Using numerous chemical, enzymatic and immunological techniques, that while the majority of the κ-casein resides on the surface of the casein micelles, other caseins might occur there as well (Dalgleish, 1998; Horne, 2006). In all models for casein structure, κ-casein is thought to predominate on the micellar surface (Hill and Wake, 1969). In milk clotting in the neonate stomach, the enzyme chymosin (rennin) specifically cleaves one bond in κ-casein to initiate aggregation of the micelles. It has been clearly demonstrated recently (Shekar *et al.*, 2006) by κ-casein gene null mutation experiments in mice, that κ-casein is essential for the assembly of the casein micelles and for lactation to occur *in vivo*.

6.2.2 The submicelle theory of casein structure

For many years, the most accepted theory (Farrell, 1988; Schmidt, 1982) of the structure of the casein micelle was that it was composed of spherical aggregates of the caseins (submicelles) held together by calcium–phosphate linkages. The submicelle hypothesis has been historically supported by biochemical and biophysical studies (Kumosinski *et al.*, 1987; Stothart, 1989; Kakalis *et al.*, 1990; Jang and Swaisgood, 1990) on the individual casein components and reconstitution of micelles from their component caseins (Griffin *et al.*, 1988; Slattery, 1979), as well as electron microscopy of the micelles themselves (Carroll *et al.*, 1968) and partially disrupted micelles. Early studies on the calcium-depleted caseins demonstrated that in the absence of calcium they formed rather large aggregates, traditionally termed as 'submicelles' (Walstra, 1990; Stothart, 1989; Pepper and Farrell, 1982; Jang and Swaisgood, 1990), and that these aggregates formed colloidal complexes in the presence of added calcium. Gradual removal of

calcium by dialysis or EDTA treatment showed the emergence of rather uniform submicellar structures, as visualized by physical techniques and freeze fracture electron microscopy (Lin *et al.*, 1972; Knoop *et al.*, 1979). These aggregated particles had physical properties similar to the aggregates found in whole casein preparations in the absence of calcium and were considered as submicellar in nature. Based on these data, Schmidt proposed the 'submicelle' model. In summary, this model begins with 'specific and productive' casein–casein interactions, traps calcium phosphate and forms the colloidal complexes.

This theory has been later challenged by concepts (Holt, 1992; Horne, 1998) arising from the study of the casein–calcium–phosphate interactions, the micelles themselves, and physical chemical studies of the structure–function relationships and calculations from polymer condensation theory.

6.2.3 Casein micelle models with an internal gel matrix

Two more recently proposed models for the casein micelle have emerged that refute the notion of discrete submicellar structures within the micelle. The first experiment to indicate departure from the submicelle theory was that of Rose and Colvin (1966) who, from X-ray diffraction, postulated that the electron-dense particles were granules of colloidal calcium phosphate instead of proteins. During a series of studies on casein–calcium–phosphate interactions, Holt and coworkers (Holt *et al.*, 1998; Holt, 1998) discovered that the phosphopeptide fraction of β-casein could bind to and stabilize calcium–phosphate aggregates, resulting in the formation of nanoclusters of a discrete size and composition: without the peptides, the calcium phosphate structures would grow randomly and precipitate. This discovery led De Kruif and Holt to propose that such nanoclusters are the centerpiece of the casein micelle structure (de Kruif *et al.*, 2002). The formation of nanoclusters with a radius of 2.3 nm would drive micelle formation by randomly binding phosphoproteins, causing an inverted micelle, and then more proteins could coat this new hydrophobic surface and, in turn, bind more calcium phosphate until a size limited colloid is formed. There are about 800 of these amorphous calcium phosphate nanoclusters in an average sized casein micelle (~100 nm in diameter). This nanocluster model is supported by the rheomorphic theory of casein structure (Holt and Sawyer, 1993). In this view, the unstructured proteins form about the amorphous inorganic species and their function of binding to the calcium phosphate gives rise to their structure; hence no specific protein secondary structures or protein–protein interactions are invoked, except that a surface position for κ-casein is required. However, it is not yet clear what is the cell assembly mechanism that causes this to happen.

The casein micelle model proposed by Horne (1998, 2002, 2006) considers the surface chemistry of the individual caseins and their assembly behavior, and concludes that protein–protein interactions are indeed

important, but in essence the model retains the rheomorphic concept. In this view, the amphiphilic nature of the caseins causes them to act more as block copolymers of alternating charge and hydrophobicity, that is, a charged phosphopeptide loop on the N-terminal and a hydrophobic train toward the C-terminal for β-casein, the reverse being true for κ-caseins, in which an N-terminal hydrophobic train is followed by a charged loop. In the case of α_{s1}-casein, it ends with a final C-terminal hydrophobic train. In this model, the hydrophobic interactions among various caseins are mostly considered, and the growth of the calcium phosphate nanoclusters begins the process of micelle formation, but it is limited by binding to the phosphopeptide loop regions of the caseins. Once bound to the amorphous inorganic matrix, further protein–protein interactions are related to the hydrophobic blocks, and polymerization proceeds by repeating the entire process. Micelle formation leads to an internal gel-like structure with embedded nanoclusters of calcium and phosphate, and the reaction of κ-casein, which contains only one phosphoserine residue, limits micellar growth by acting as a dead-end capping unit, in analogy with the growth of synthetic polymers.

Interestingly, these two distinctly different views of the internal structure of the casein micelles–submicelles versus gel matrices arise essentially from the same biochemical and physical chemical databases. For a further exposition on their similarities and differences, see the excellent review of Pieter Walstra (1999). A recent review by McManhon and Oommen (2008) suggested an interlocked lattice model (to reconcile the combined action between casein-calcium phosphate aggregates and casein polymer chains as the main building block) that maintains the integrity and stability of the casein micelles.

6.3 Synthesis and secretion of caseins

6.3.1 Cell physiology

The evolution of the mammary gland, presumably from external sweat glands, has yielded a great variety of exterior appearances in many species (Patton, 2004), but at the tissue level there is a common organizational theme as shown in Fig. 6.2a. Mammary secretory cells are epithelial in nature and are arranged in alveoli which are connected to ductal tissue. The secretory epithelial cells (SEC) are surrounded by a layer of myoepithelial cells, which are able to contract and expel milk into the ducts in response to the hormone oxytocin. The alveoli are highly vascularized to ensure a constant flow of the metabolic precursors needed for milk synthesis and secretion. Finally the vascularized alveoli are embedded in an extracellular matrix. This matrix not only supports the cells, but also, through cell–cell interactions, is responsible for the full expression of the genes that control milk synthesis (Patton, 2004).

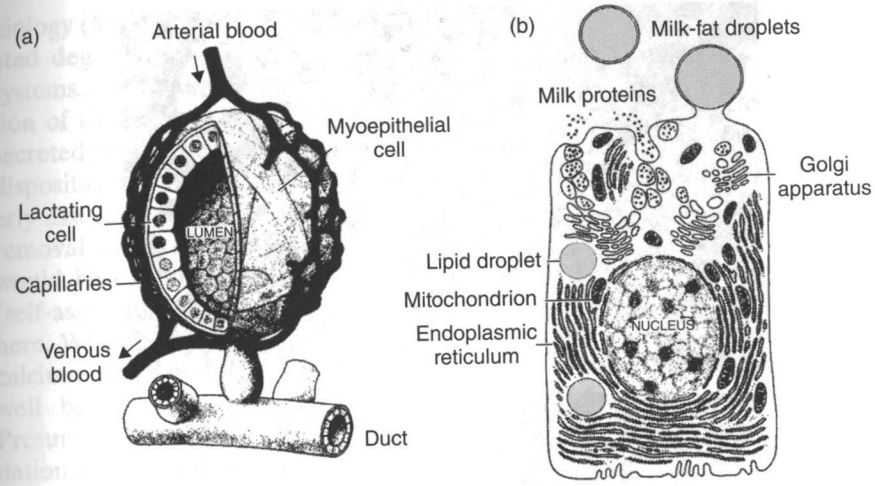

Fig. 6.2 Cell physiology of lactating mammary gland. (a) A single alveolus consisting of lactating epithelial cells (SEC) surrounding the lumen. (b) A typical lactating cell indicating active secretion of protein and lipid by distinct mechanisms; reprinted with permission of *Scientific American* and S. Patton.

6.3.2 Protein synthesis and secretion: Overview

Adaptation of milk components to their ultimate aqueous environment begins during secretion. Lipid and protein synthesis are partitioned from the start. Amino acids and their metabolic precursors are actively transported into the SEC and assembled into proteins on the ribosomes of the highly developed rough endoplasmic reticulum (Patton, 2004). All milk proteins of mammary origin have conserved leader sequences which cause insertion of the nascent proteins into the lumen of the endoplasmic reticulum (ER), shown in Fig. 6.2b. The proteins are then transported through the Golgi apparatus (Patton, 2004; Farrell, 1988), as shown in Fig. 6.2b; presumably the globular proteins of milk are folded during this period. In the Golgi apparatus, the caseins, which are the major milk proteins in most species, appear to be spherical complexes of about 10 nm in diameter. The caseins are phosphorylated by a calcium-activated membrane-bound kinase to begin the process of calcium transport (Bingham and Farrell, 1974). A membrane associated ATPase delivers calcium to the vesicles (Bingham *et al.*, 1993). The gradual intercalation of calcium, casein, and phosphate into the submicellar structures (or complexes formed through specific casein–casein interactions) leads to the formation of casein micelles and insures the effective transport of these vital minerals. This process can be visualized in Fig. 6.3 (top), where small submicellar or particles of casein–casein complexes are seen in the secretory vesicles nearest the trans Golgi. Through the binding of calcium and the accretion of phosphate, the

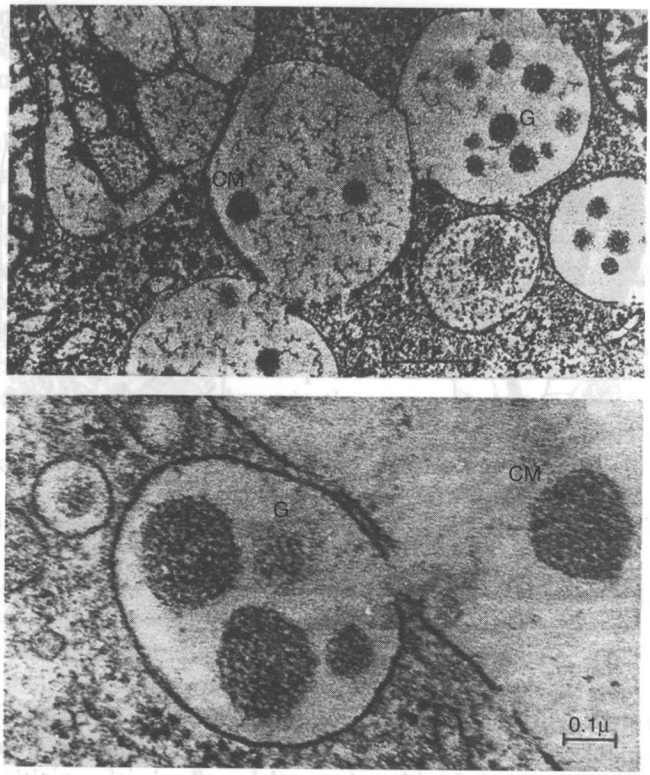

Fig. 6.3 Formation of casein micelles (CM) within Golgi vesicles (G) and depicting the aggregation of small submicellar particles into large micelles (top). A Golgi vesicle (G) about to discharge its contents into the alveolar lumen (bottom); a casein micelle (CM) is already present in the lumen; reprinted from Farrell *et al.* (2006a).

colloidal casein micelles are formed and finally secreted by reverse pinocytosis (Fig. 6.3, bottom). Overall, this view strongly supports the involvement of proteinaceous complexes in the synthesis and secretion of casein micelles though it remains unclear if any structural rearrangements or changes occur during and after secretion.

6.3.3 Protein synthesis and secretion: Details in the ER lumen

The process of casein secretion within the lumen of the ER has not been studied in specific detail (Farrell *et al.*, 2006a); however, reference to recent information on cell biology and protein folding in other systems may shed more light on the issues of casein micelle formation. The issue of quality control of protein folding has become a widely researched area in cell

biology (Meusser *et al.*, 2005). The process of endoplasmic reticulum associated degradation (ERAD) has been found to occur in many secretory systems. As noted above, conserved leader sequences of casein cause insertion of the nascent proteins into the lumen of the ER. Before the newly secreted proteins can traffic beyond the ER, they must fold into their final dispositions, and multicomponent systems must assemble. Failure to properly associate or fold leads to the unfolded protein response, tagging and removal by the ERAD system. Overall, the environment of the ER lumen would be conducive to the signature functional property of the caseins (self-association) so initial casein–casein interactions would naturally occur here. While the ER lumen serves as a calcium storage center, the free calcium ion concentration fluctuates between 1 and 3 μM, a concentration well below the binding constants for the unphosphorylated caseins. Presumably, proper association of the caseins helps them to escape degradation and move on to the Golgi for processing. It has been our contention that conserved sequences of the individual caseins give rise to selected secondary structural elements and that these elements lead to self-association of the caseins without classical protein folding (Farrell *et al.*, 2002b, 2003a): that is to say, caseins contain little or no tertiary structure and proceed directly from secondary structure formation to quaternary structures exhibiting both rigid and flexible elements. This is in line with the basic tenet of structural biology that protein structure gives rise to biological function – the Anfinsen hypothesis (Anfinsen, 1973). Recent development in the field of protein folding has shed light on understanding protein self-assembly and aggregation as delineated by Jaenicke and Lilie (2000). Self-associations are those on-line and productive reactions which lead to competent biological protein assemblies such as amyloid (Jahn *et al.*, 2006), whereas non-productive aggregations lead to often mis-assembled protein complexes. The latter would be targeted for the ERAD process. The question now arises as to which of the many known *in vitro* reactions of the caseins studied to date are important in this context. It may be of interest to examine those 'productive' self-associations that may lead to the formation of casein micelles and those 'non-productive' aggregations formed through various protein–protein interactions.

The self-association of bovine β-casein has been studied by many (for example de Kruif and Grinberg, 2002; O'Connell *et al.*, 2003). The most commonly accepted mechanism is that the β-casein molecules form rather spherical polymers of limited size, following a critical micelle pathway. Interestingly, the dimensions of this polymer are rather fixed, but its molecular weight is highly dependent on ionic strength and temperature. In species such as human, where β-casein is the predominate protein, this process could be viewed as a prominent on-line self-association in the ER lumen. It must be noted that the unphosphorylated form of human β-casein has nearly the same propensity for self-association as its phosphorylated forms (Bu *et al.*, 2003). The weight average molecular weight of bovine

Table 6.2 Weight average molecular weights of selected caseins and mixtures by analytical ultracentrifugation at 37°C

Casein or mixture	Weight average molecular weight	Weight average polymeric size	Rotor speed (rpm)
α_{s1}-Casein[b]	56000	Dimer	12000
β-Casein[c]	1250000	52 mer	3000
RCM κ-Casein[d]	3040000	160 mer	3000
1.5 α_{s1}-:1 RCM κ-[d]	316000	15 mer	3000
4 α_{s1}-:1 RCM κ-[c]	92400	Tetramer	6000
4β-:1 RCM κ-[c]	1010000	43 mer	3000
1β-:1α_{s1}-[c]	213000	Nonamer	3000
RCM whole casein[c]	110000	Hexamer	6000

[a] All data were obtained at 37°C, pH 6.75 in 25 mM PIPES (disodium piperazine-N,N′-bis(2-ethane sulfonic acid)) with 80 mM KCl to mimic milk salt conditions in the mammary gland in the absence of calcium: The rotor speeds were appropriate to the weight average molecular weight as previously described (Malin *et al.*, 2005; Farrell *et al.*, 2003b). The protein SH groups were reduced and carboxymethylated (RCM).
[b] (Malin *et al.*, 2005).
[c] (Farrell *et al.*, 2006a).
[d] (Farrell *et al.*, 2003b).

β-casein at 37°C in the absence of calcium is 1250000, as shown in Table 6.2. This is almost three times the estimated value for the submicellar structures (or casein–casein complexes) and could potentially lead to ER stress and ERAD tagging.

Studies on the polymerization of α_{s1}-casein had previously shown (Alaimo *et al.*, 1999) that the molecule exhibits a progressive consecutive association to dimers, tetramers, hexamers, etc., and that this process is highly dependent on pH and ionic strength. Most early studies (Schmidt, 1982) on the polymerization of α_{s1}-casein were conducted at or below 25°C. Because of its hydrophobic nature, it was expected that polymerization would be accentuated at 37°C. This clearly is not the case, as shown in Table 6.2: all three major genetic variants depolymerize (Malin *et al.*, 2005) and behave essentially as dimers. The dimer formation is centered on its C-terminal half, with a strong selective interaction between residues 136 and 160 of each monomer, and there appears to be no involvement of N-terminal hydrophobic train of α_{s1}-casein at normal ionic strength and 37°C. In addition, the latter region is positively charged and could participate in phosphate binding. This appears to contradict one of the modes of crosslinking postulated by Horne (1998) as part of the casein gel network. Finally, it has been shown (Bingham *et al.*, 1972) that both native and dephosphorylated α_{s1}-caseins undergo similar aggregation and precipitation reactions. Thus, in the ER lumen, the α_{s1}-casein molecule would not be greatly polymerized, and the higher order polymers observed *in vitro*

are the products of an aggregation process, which would likely be 'non-productive'.

Of all the caseins, the self-association behavior of α_{s2}-casein has been studied the least (Euston and Horne, 2005). Horne (1998) has speculated that it will form linear micelles similar to α_{s1}-casein.

κ-Casein is the calcium-soluble stabilizing protein of the casein micelles: it is also the only casein whose disulfide bonds play a significant role in casein structure. As isolated from milk, the protein displays a unique laddered disulfide bonded pattern in SDS gel electrophoresis, with sizes ranging from dimer to octamer and above (Farrell *et al.*, 2003b). In analogy with the use of cleavable disulfide reporter groups, it can be stated that the nearest neighbor to a κ-casein molecule is another κ-casein molecule, as shown in the Schmidt model (Schmidt, 1982). In essence, the monomeric κ-casein molecules depicted in the Horne model (Horne, 1998) are not found.

The source of the laddered disulfide pattern of κ-casein is unknown. In most secretory systems, the enzyme protein disulfide isomerase (PDI) occurs on the inner membrane of the ER, and acts as both a chaperone and a catalyst for the rearrangement of disulfide bonds. However, this enzyme may not be responsible for the linear polymer pattern. For this pattern to form, the κ-casein monomers would have to be in a queue at or near the PDI. Theκ-casein's ability to polymerize may inhibit the action of PDI and thus allow it to retain the SH character in the ER. Therefore, the studies on the polymerization of reduced κ-casein that demonstrate an association involving a critical micelle model similar to that of β-casein (Schmidt, 1982) may be relevant. However, as was the case for α_{s1}-casein, these studies were primarily conducted below 25°C. At 37°C, reduced carboxymethylated κ-casein (RCM-κ) forms large, stranded amyloid bodies (Farrell *et al.*, 2003b); the latter, as seen in Table 6.2, are clearly aggregations and not a part of competent casein secretion. So the κ-casein must either be SH capped or self associate with other caseins for the successful transit through the ER lumen. At a ratio of 1.5 α_{s1}-:1 RCM κ-casein, amyloid formation is inhibited and moderate molecular weight complexes of 316000 are formed at 37°C (Table 6.2). Increasing the ratio of α_{s1}-casein to 4:1 substantially reduces the complexes to 92400. Bovine β-casein, while limiting amyloid formation, does not have the same effect of reducing the weight average molecular weight as α_{s1}-casein (Table 6.2). Since the κ- and β-caseins seem to share a similar self-association mechanism, it would appear that in mixed associations the reduced κ-casein can be inserted interchangeably into the self-association reaction of β-casein, but the resulting overall size is somewhat smaller than that of β-casein alone (Table 6.2). Such large complexes might not allow the associated proteins to escape ERAD and move on to the Golgi apparatus. In fact, Chanat *et al.* (1999) have studied the transport of caseins from the ER to the Golgi apparatus in mammary epithelial cells. Their data suggest that for animals with high casein content, α_{s1}-casein must

interact with the other caseins for efficient transport to the Golgi. In cells that completely lack α_{s1}-casein, the accumulation of β-casein (or κ–β mixtures?) is observed in the ER. In the long term, this causes ER stress, activates the ERAD system and impedes secretion. Recent work by Shekar *et al.* (2006) has demonstrated that κ-casein is essential for the formation of casein micelles, as well as for lactation to occur.

To test the efficacy of α_{s1}-casein at reducing the size of β-casein aggregates *in vitro*, 1:1 mixtures of the two proteins were studied by analytical ultracentrifugation at 37°C. The weight average molecular weights of the complexes were speed dependent, increasing with decreasing speed, indicating strong hydrophobic interactions; see Table 6.2. The weight average molecular weight of the 1:1 complexes was 213000, which represents a six-fold reduction of the β-casein aggregate or a four-fold increase over that of the α_{s1}-casein alone. This result is of importance because there are few studies of the mixed associations of these two caseins at 37°C and conditions close to that of the ER. Here, the α_{s1}-casein acts to diminish the size of either the β- or κ-casein aggregates; in this sense it may be considered a molecular detergent for the other caseins. Thus, these *in vitro* data confirm the *in vivo* observations that α_{s1}-casein can reduce aggregated species and allow the associated particles to escape the ER. For human milk, the small amount of α-caseins present may help reduce the aggregates of β-casein: also, the net casein content in human milk is only 17% of that in bovine milk (Table 6.1), so smaller aggregates would be favored (Dev *et al.*, 1994, Sood *et al.*, 1997). Finally, RCM-derived whole bovine casein, with the standard ratios of the four caseins, has a weight average molecular weight of 110000 at 37°C under the same conditions of pH and in the absence of calcium. From all of these biological and physical chemical studies it would appear that for the competent synthesis and secretion of casein: preformed casein complexes of the size of the putative casein submicelles (or casein–casein complexes) must form through protein–protein interactions – triggered by conserved protein sequences – and emerge from the ER for efficient transit via secretory vesicles to the Golgi apparatus.

6.3.4 Protein synthesis and secretion: Details in the Golgi vesicles

From the discussion above, it seems most likely that the individual casein molecules undergo significant self-association in the ER and are then transported in vesicles to the cis face of the Golgi apparatus. In this region, three significant events occur in the process of casein secretion. The first event is most likely an increase in calcium concentration, accomplished by an ATPase-driven pump (Bingham *et al.*, 1993). The second most likely next step is the phosphorylation of the associated caseins by a membrane-associated casein kinase which uses calcium-ATP as substrate and is specific for Ser residues, preceded at the n + 2 position by Glu or a serine phosphate (SerP) residue (Farrell *et al.*, 2004). The casein kinase (Bingham and Farrell,

1974) responsible for this reaction has not been purified, but in Golgi preparations the enzyme requires a surprisingly high calcium ion concentration (K_M~20 mM) so that, at the time of phosphorylation, calcium ions may be almost immediately bound to the caseins (K_D~5 mM). The third and often overlooked step is that one of the byproducts of the kinase reaction, ADP, retains bound calcium, so that when this is converted by membrane associated diphosphatases to phosphate and AMP (Farrell *et al.*, 1992), both calcium and phosphate are released near the interior membrane surface where the casein proteins are still being phosphorylated. Studies by West and Clegg (1983) showed that phosphorylation of casein is still proceeding in large Golgi vesicles as is, most likely, calcium transport. Thus, both calcium and phosphate may automatically be bound to the casein–casein complexes, as seen in Fig. 6.3, prior to micelle formation. In general, when milks that contain >2% protein are analyzed, the accompanying inorganic phosphate and calcium levels found, yield insoluble precipitates (apatite or brushite) in the absence of casein. But the question arises, are the concentrations of these compounds ever high enough or concentrated enough to form nanoclusters in the Golgi vesicles? Additionally, would the energy gained by coating these precipitating clusters be sufficient to depolymerize the preformed casein complexes in the manner suggested by the Horne model? Veis (2005) has suggested that, in general, mineralization in mammalian systems such as collagen and dentin matrices is directed and controlled (assembled) by the structural proteins present: inorganic direction appears to be limited to simpler systems such as the crystalline shells of corals.

Based on the average composition of the colloidal caseinate (Farrell, 1988), the average concentrations of calcium and phosphate within the colloidal complexes are 18.7 and 15.2 mM, respectively. In turn the average concentration of casein within the casein micelles is 1 mM, but the average casein molecule has 6.5 phosphate groups, thus the concentration is 6.5 mM SerP. Moreover, all four caseins have selected areas of positive surface (Swaisgood, 2003) which may bind phosphate after calcium binds to the protein as suggested for α_{s1}-casein (Malin *et al.*, 2005). In most of the models available on the structure of the casein micelles, the binding details of phosphate have been largely neglected. The prominent positive patches of the caseins are: 1–10 of α_{s1}- (+6); 165–199 of α_{s2}- (+11); 97–113 of β- (+6) and 97–116 of κ-casein (+6); these, too, average out to 6.5 mM. Assuming each serine phosphate binds one calcium, which in turn binds one inorganic phosphate, this would result in a double layer. Further, assuming each positive charge in the above mentioned areas then binds one phosphate and one calcium in a second double layer, then the possible concentrations of unbound calcium and phosphate within the micelle is further reduced. It can be seen in a molecular dynamics study (Farrell *et al.*, 2002a) that both the smaller calcium ions and the larger chloride ions bind to the peptide and then associated ions tend to form a charged double layer about the

peptide chain. In this case there is limited attraction between the aqueous calcium and chloride ions, but complexes between calcium and phosphate would be more numerous. *In vivo*, the formation of such a double layer of calcium phosphate would reduce the unbound (uninfluenced) concentrations of these two ions to 5.7 and 2.2 mM respectively. This is far from the concentrations used by Holt and coworkers (Holt *et al.*, 1998, Holt, 1998) to form nanoclusters in vitro (37 mM calcium, 30 mM phosphate and 3 mM phosphopeptide). In this same context the supramolecular aggregate would have 65 negative charges (Malin *et al.*, 2005) due to clusters of SerP groups and 65 positive charges due to clusters of basic amino acids: in total it could carry 260 ions as a simple double layer. The standard nanocluster has about 355 ions at its core (Holt *et al.*, 1998; Holt, 1998). The coalescence of two casein–casein complexes, with their bound (influenced) ions, would then terminate any possible calcium phosphate growth and begin micelle formation. It may be that nanoclusters then have an interesting and informative chemistry but actually represent a process which is an inorganic aggregation similar to amyloid formation by κ-casein, rather than an on-line productive biological process. Thus, from the point of view of the synthesis and secretion of casein micelles, the Schmidt model may be representative of the biological process, although the stoichiometry of the inorganic 'cement' is probably incorrect, based upon the latest physical chemical data (de Kruif and Holt, 2003; Dalgleish *et al.*, 2004), which indicates that a type of apatite is the most likely candidate for the molecular structure within the micelles.

6.4 Studies on the structure of casein micelles

Clearly, the proteinaceous complexes, formed through specific casein–casein interactions with an average size of ~10 nm, not necessarily spherical (Figs 6.1 and 6.3), play a major role in the on-line formation of casein micelles in mammary tissue. Additionally, it would appear that α_{s1}-casein acts as a type of detergent to limit the size of these submicellar particles in order to defeat the unfolded response and escape the ERAD system. The formation of these controlled aggregates (productive association) allows for and facilitates transfer from the ER to the Golgi apparatus. Once present in the Golgi, presumably, micelle formation subsequently occurs. Past research on understanding the detailed structure of the micelles has centered on using electron microscopy, neutron scattering and X-ray scattering techniques. Atomic Force Microscopy (AFM) (Uricanu *et al.*, 2004; Gebhardt *et al.*, 2006) has only recently been applied to the study of the casein micelles.

6.4.1 Electron microscopy

Electron microscopy (EM) represents a powerful tool for elucidation of biological ultrastructures, as seen in Figs 6.1 and 6.4. The problem with this

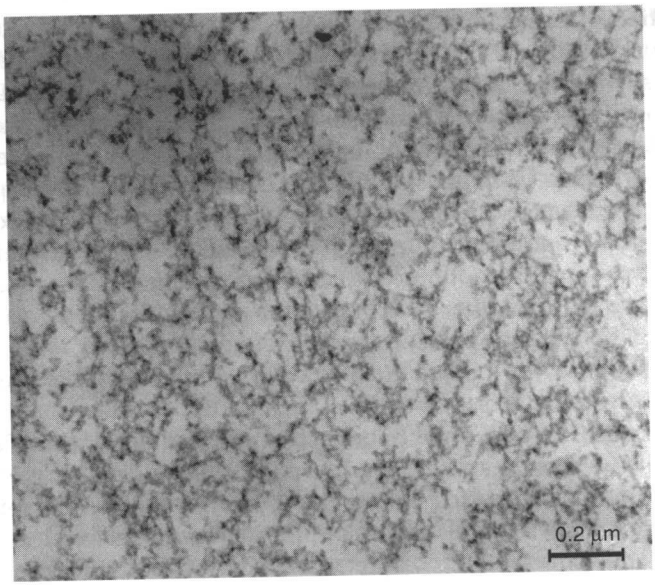

Fig. 6.4 Transmission Electron Micrograph of sodium caseinate in imidazole buffer (pH 7.0), fixed with glutaraldehyde and stained with uranyl acetate and lead citrate.

technique lies in the fixatives and metal staining used to accentuate the particular features they react with and visualize, usually at the expense of other features. In contrast, when uranyl oxalate is used as a positive stain for proteins (McMahon and McManus, 1998; McMahon and Oommen, 2008), a more uniform distribution of material is seen because the stain is binding to the caseins, particularly the SerP, and accentuating the protein distribution. However, more details can be visualized from our studies of casein micelles and sodium caseinate (Figs 6.1 and 6.4). Similar strand-like protein structures with a 'knot' about 10 nm (diameter) can be found in skim milk (Fig. 6.1) and sodium caseinate, a casein product depleted of calcium. In addition, Fig. 6.3 shows the presence of casein–casein aggregates in rat Golgi vesicles. Clearly, EM images are influenced by the stains used in the experiments. One is tempted to employ the scientific dialectic here and say that neither the thesis (submicelles) nor the antithesis (no submicelles) is correct but that synthesis is needed. Walstra (1999) has proposed that the submicelles re-emerge in EM representations of products such as cheese. Work on a variety of cheeses (Tunick *et al.*, 1997) demonstrates the dynamic nature of the submicellar structures, loosely defined as casein–casein complexes of the cheese protein matrix.

6.4.2 Micelle dissociation studies

Studies on micelle dissociation were among the first to indicate the existence of submicelles or casein–casein complexes, and Schmidt (1982) drew heavily on these and on the reconstitution studies (Schmidt and Payens, 1976). Clearly, the Walstra hypothesis on micelle equilibria is at play in these experiments (Walstra, 1999). Those components in rapid equilibrium will quickly exchange and yield one result, while those slow to equilibrate will accentuate another feature. It has also been suggested that calcium binding to caseinates must precede phosphate binding (Visser *et al.*, 1979). Temperature plays another role in that the aggregation of α_{s1}-casein is, as noted above, accentuated at lower temperatures.

Finally, Holt (1998) studied the effect of κ-casein on micelle dissociation: he expected that added κ-casein would cause dissociation of the micelles, and it did not. The experiment conducted was similar to what Talbot and Waugh (1970) termed micelle transformation. In all previous studies of κ-casein content versus size, the more κ-casein present, the smaller the micelles (Sood *et al.*, 2003). Addition of purified κ-casein to micelles causes a shift to smaller sizes, not complete dissociation. It should also be noted that the purified κ-casein used in these experiments represents an SH-capped laddered polymer and not a reactive reduced monomeric species as discussed above (Table 6.2).

6.4.3 Scattering studies

Both small-angle X-ray scattering (SAXS) (Kumosinski *et al.*, 1988; Holt *et al.*, 2003; Marchin *et al.*, 2007; Pignon *et al.*, 2004) and small angle neutron scattering (SANS) (Stothart and Cebula, 1982; Hansen *et al.*, 1996) have been applied to the casein micelles. Both these techniques provide information on the electron density of the sample relative to the solvent. Because of technical limitations regarding the wavelength of the radiation relative to the total particle size, SAXS methodologies, in essence, provide a viewing window on the micelles. The data then must be interpreted in terms of the density of the average particles observed within the micelles. To circumvent this problem it has been common to study first the sodium caseinate (protein aggregates), which is totally contained within the experimental q-scale, and so determine its scattering density. Comparison of the density difference is then made with that observed for the window on the micelle. When this is done carefully, good inferences into the nature of the particles within the overall micelle structure can be made. Using these concepts and enhanced experimental techniques, the two SANS studies came to very similar conclusions that the micelles have within them particles with electron densities (scattering centers) similar to those found for the sodium caseinates. For the SAXS data, the electron density difference for the sodium caseinate is extremely low relative to globular proteins (9.9 e/nm^3 for casein versus 67 e/nm^3 for α-lactalbumin) and the particles within the micelles have this same

low electron density: similar calculations can be done for the SANS data. Where these calculations differ is in the mathematical models used to fit the data for the proteins. Basically, the scattering centers within the micelles display a good deal of heterogeneity leading Hansen *et al.* (1996) to conclude a polydisperse distribution of submicelles, while Stothart and Cebula (1982) postulated submicelles of a more closely packed nature. Kumosinski *et al.*(1988) fitted their data for sodium caseinate to a somewhat lopsided sphere within a sphere: basically, a spherical hydrophobic core and a loose hydrophilic shell reminiscent of Schmidt's submicelle model (Schmidt, 1982). However, the data could be fitted well only for reformed synthetic micelles when there was significant overlap among the casein molecules contained in adjacent submicelles. These studies arrived at the same conclusion: proteinaceous complexes formed through specific casein–casein interactions exist within the casein micelles.

Holt *et al.* (1998) studied calcium phosphate nanoclusters with both SANS and SAXS, and this led them to speculate that the calcium phosphate 'clusters rather than putative submicelles could be solely responsible for the heterogeneous structure revealed by electron microscopy, neutron scattering and X-ray scattering.' Recent studies on casein micelles by Marchin *et al.* (Pignon *et al.*, 2004; Marchin *et al.*, 2007) using SAXS/USAXS, combined with the more modern microscopic technique of cryo-TEM, also questioned the existence of submicelles and suggested that the micelles are dominated by calcium phosphate nano-clusters of 2.5 nm in diameter, which is in general agreement with Holt's model. However, the details of the internal structure and subsequent reorganization upon the removal of serum calcium phosphate or of β-casein remain uncharacterized.

Arguably, we raise the prospect that casein–casein interactions might be as prevalent as the possible existence of inorganic calcium phosphate nanoclusters within the micelles. Despite the fact that the debate over the molecular details involving the formation and stabilization of casein micelles is likely to continue, few would doubt the complex and dynamic nature in the delicate balance of hydrophobic (casein–casein) interactions and electrostatic (casein–casein and casein–calcium–phosphate) interactions. The fast advancement in the field of protein folding research, theoretical and experimental, combined with emerging bioinformatics methodologies will, without doubt, significantly advance our understanding of the growing class of proteins such as casein which inherently lack a well-defined three-dimensional (3D) structure.

6.5 Future trends

Past research on casein micelles has provided us with a wealth of information for consideration of possible applications aimed at improving human health. Various attributes of casein micelles, including their size and relative stability, make them an ideal candidate for the currently ever-growing

arena of nanotechnology. It has recently been demonstrated (Semo *et al.*, 2007; Sozer and Kokini, 2009) that casein micelles may be manipulated to act as an effective delivery vehicle for minerals, vitamins, bioactive and nutraceutical compounds through nanoencapsulation and nanoparticulation processes. The lack of unifying safety regulations and generally negative public perception of manufactured nanomaterials for food applications will undoubtedly highlight the natural and superior advantages of casein micelles.

6.6 Acknowledgements

The author would like to thank Dr Harold M. Farrell, Jr. for fruitful discussions and acknowledge Dr Peter H. Cooke for his technical assistance in obtaining the high resolution TEM images presented in this work.

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